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ORIGINAL ARTICLE

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Salivary analyses and caries increment over 4 years: an approach by cluster analysis

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Abstract *Main problem.* Caries as a multifactorial process is influenced by salivary defense. Cluster analyses should give additional information on the role of salivary variables in relation to caries increment. *Methods.* Samples of unstimulated and stimulated whole saliva from 28 young adults (mean age 23.5±2.1 years) were analyzed for flow rate, pH and buffer variables, lysozyme, lactoferrin, peroxidase, thiocyanate, secretory immunoglobulin A, and total protein. The decayed, missing, and filled surfaces (DMFS) were recorded at baseline and after 4 years. Cluster analyses were executed on the basis of salivary data. *Results.* The mean caries increment (ΔDMFS) over 4 years was 6.7±4.0 (range 1–16). In two-cluster processing, three out of four volunteers with low caries increments were grouped into one cluster. Only a few variables proved to be important for cluster characteristics. *Conclusions* The results suggest that over 4 years (1) the volunteers with very low caries increment (ΔDMFS=1) are classified always together, (2) these volunteers do not form a separate cluster by themselves, (3) low caries increment was related to higher salivary flow rate and lower levels of lysozyme and lactoferrin for unstimulated saliva and (4) the partial pressure of CO_2 was of importance in stimulated saliva.

Keywords Caries decay · Caries increment · Cluster analysis · Defense factors · Saliva

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Introduction

Human saliva contains several factors with protective or antibacterial properties in the oral cavity [29]. Usually these factors are divided into immune and nonimmunoglobulin agents. While the immunoglobulins (Ig) A, G, and M form the first part, peroxidase, lysozyme, agglutinins, and lactoferrin are examples of innate defense factors [26]. These factors have been widely studied in vitro. The relationship between caries and microbiological or defense factors in the saliva has been reported in cross-sectional and to a lesser extent in longitudinal studies [15, 20, 32]. A longitudinal study exists on human salivary antimicrobial agents and caries increment over 2 years [15] also considering a cluster analysis. Longitudinal studies considering salivary variables have been executed predominantly in children or adolescents [2, 6, 9].

Salivary IgA, which predominantly is secretory, constitutes the main specific immune defense mechanism in saliva [18]. Elevated levels of secretory IgA have been found together with higher numbers of decayed surfaces in adolescents [25], and anti-*Streptococcus mutans* IgA antibodies were reduced in the unstimulated saliva of children with protein-energy malnutrition [14]. Lactoferrin in the saliva was reduced by the use of smokeless tobacco, but the DMFS scores were similar to those of nonusers [11]. A significant relationship between lactoferrin and caries lesions was found in adolescents [25]. The output of salivary peroxidase, lysozyme, and other nonimmune factors is decreased in patients treated with beta-2-adrenoceptor agonists, and significantly higher numbers of initial and manifest caries lesions were found

in them [23]. Peroxidase in saliva catalyzes the conversion of thiocyanate to hypothiocyanite, which is regarded as having a regulatory role in caries incidence [27, 28].

While cross-sectional studies on the relationship between salivary factors and caries often were controversial or failed to demonstrate any relationship [3], longitudinal studies are regarded as more important [1]. One possible explanation for the inability to find a straightforward relationship between saliva and dental caries could be the inappropriateness of caries incidence or caries increment as accurate measures of caries activity. Therefore, it seems valuable to group subjects by salivary data and investigate the distribution of caries data among these groups.

The aim of this study was to (1) execute a longitudinal study over 4 years in young adults regarding caries increment and (2) try a multifactorial modeling by cluster analyses using salivary factors and their relationship with caries increment over 4 years. No study using salivary cluster analysis for this topic over such a long time period could be found in the literature.

Materials and methods

Twenty-eight volunteers, 12 males and 16 females (mean age 23.5±2.1 years old at the start) with different caries decay, participated in the study after approval by the Ethics Commission of the Rostock University Faculty of Medicine. Criteria for inclusion were no general diseases, no medication, and no partial dentures or bridges. All participants were examined clinically and by bite-wing radiographs at baseline and after 4 years. The DMFS indices were recorded in accordance with the World Health Organization guidelines [34]. Accordingly, D1 lesions were not included in the D index but registered apart. Then the caries increment (ΔDMFS) over 4 years was calculated.

Unstimulated and masticatorily stimulated whole salivas were collected in tubes on ice under standardized conditions between 7:00 AM and 8:00 AM at baseline. Unstimulated saliva was collected for 15 min by drooling. Stimulated saliva was collected for 5 min by chewing on paraffin wax and spitting intermittently. No oral hygiene or breakfast were allowed in the morning before saliva collection. The flow rates as well as pH, buffer effect, and buffer variables (partial pressure of CO_2, bicarbonate, total buffer bases) were determined immediately after collection of saliva. Then the salivary specimens were centrifuged at 3000 *G* and 4°C for 15 min and the supernatant was used for biochemical analysis. Peroxidase activity was estimated on the same day, and aliquots of the remaining salivary supernatant were stored at −18°C for 10 weeks. After thawing, it was analyzed for thiocyanate, lactoferrin, lysozyme, total protein and secretory IgA (sIgA). The flow rate was calculated as ml/min, and pH was determined electrometrically with a MGA100 MKC pH meter (Meinsberg, Waldheim, Germany). The buffer effect was determined by the final pH after a challenge with 0.003 M and 0.005 M HCl, respectively [7]. The same pH meter was used. The partial pressure of carbon dioxide as well as the concentrations of bicarbonate and total buffer bases were determined using the AVL 945 automatic blood gas system (AVL, Roswell, USA). The concentration of thiocyanate was determined as described by Betts and Dainton [4] with ferric ion in nitric acid. The determination of salivary peroxidase was performed according to Chance and Maehly [5] with guaiacol used as the substrate, oxidized by hydrogen peroxide. The spectrophotometric analysis was performed at a wave length of 470 nm. Since the guaiacol is not converted stoichiometrically, a unit U* is defined here as the enzyme activity causing an absorbance change of extinction (ΔE) of 1.0 per min. Lactoferrin was analyzed using an enzyme immunoassay [30].

After coating the tube walls with lactoferrin and washing, a polyclonal peroxidase-labeled antibody to lactoferrin was added. After a further washing, the peroxidase was reacted with o-phenylenediamine as the substrate. The absorbance was measured at a wavelength of 405 nm. Lysozyme was determined using the lysoplate method [19]. Plates with agarose gel supplemented with lyophilized material of *Micrococcus luteus* (Boehringer, Mannheim, Germany) were inoculated with the samples or calibration solutions, respectively, and incubated at 37°C. After 24 h, the diameters of the lysed areas were measured. Hen egg white lysozyme (HEWL) (Boehringer) was used as a standard. Thus, lysozyme activity is expressed here relative to the activity of HEWL of the indicated concentration. The protein content was determined according to Lowry et al. [17] using human serum albumin as a standard. Secretory IgA was determined by enzyme immunoassay [24] using a monoclonal antibody against the secretory component in the first binding step and a polyclonal peroxidase-labeled antibody against IgA in the second step. Colostral sIgA served as a standard.

Statistical analysis was performed using SPSS software for Windows. Hierarchic cluster analyses were performed based on the data calculated as concentration or activity, except for variables of unstimulated and stimulated saliva, respectively, and then together for unstimulated and stimulated saliva. The agglomerative Ward method was applied to calculate the quadratic euclidean distance and unify objects with the greatest similarity. Then, the mean values and standard deviations of the selected salivary variables were calculated for every cluster. The *t*-test comparing the composition of the clusters was performed at a significance level of $P \leq 0.05$.

Results

The clinical results at baseline are given in Table 1. The mean value for DMFS was 32.6. One additional caries lesion per year was taken as the threshold for high or low caries increment. Accordingly, the volunteers over 4 years were divided into a group with low caries increment (ΔDMFS≤4), including eight volunteers, and a group with high caries increment (ΔDMFS>4) including 20 participants. No volunteer had ΔDMFS=0 during the 4-year period. Of the eight volunteers with low caries increment, three presented a very low increment of ΔDMFS=1 during these 4 years.

Considering the input of all variables of unstimulated saliva, a two-cluster model classified all three volunteers with ΔDMFS=1 together in one cluster but, besides one volunteer with a low caries increment, this cluster also included 14 volunteers with caries increments of ΔDMFS>4. The distribution of volunteers is given in

Table 1 Clinical data at baseline and increments over 4 years

Variable	Mean	SD
Baseline data		
DMFT	13.5	4.2
DMFS	32.6	15.7
DS	6.7	4.0
D1S	6.4	3.2
Increment over 4 years		
ΔDMFT	1.8	1.4
ΔDMFS	6.7	4.0

Table 2 Distribution of groups of volunteers in the cluster analyses

	Cluster 1	Cluster 2
Mean DMFS	30.8	33.6
Total volunteers	18	10
Volunteers with ΔDMFS≤4	4	4
Among them: ΔDMFS=1	3	0
Volunteers with ΔDMFS>4	14	6

Table 3 Mean values of salivary variables without significant group differences at cluster modeling (n=28)

Variable	Mean	SD
Unstimulated saliva		
pH	6.78	0.22
Final pH with 0.003 M HCl	4.40	0.83
Final pH with 0.005 M HCl	3.28	0.56
pCO_2, kPa	2.92	0.93
Buffer bases, mmol/l	14.09	3.43
Bicarbonate, mmol/l	4.28	1.41
sIgA, mg/l	18.08	20.62
Peroxidase, U*/ml	1.41	1.37
Total protein, g/l	1.22	0.96
Stimulated saliva		
Flow rate, ml/min	1.78	0.61
pH	7.11	0.20
Final pH with 0.003 M HCl	6.38	0.94
Final pH with 0.005 M HCl	3.28	0.56
Buffer bases, mmol/l	30.67	8.36
Bicarbonate, mmol/l	11.38	3.73
Lysozyme, mg/l	6.77	6.75
sIgA, mg/l	5.33	4.19
Peroxidase, U*/ml	1.33	1.08
Thiocyanate, mmol/l	1.53	0.65
Total protein, g/l	0.87	0.68

Table 2. No significant differences between clusters 1 and 2 were found for pH, final pH with 0.003 M HCl and 0.005 M HCl, buffer bases, pCO_2, bicarbonate, sIgA, peroxidase, and total protein. Mean values for these salivary variables are given in Table 3.

Significant results of salivary analysis for both clusters are presented in Table 4. Significant differences between both clusters could be found in unstimulated saliva for salivary flow rate (P=0.008), lysozyme (P<0.001), lactoferrin (P=0.028), and thiocyanate (P=0.003). Here the volunteers with very low caries increment were classified into the cluster with higher salivary flow rate, lower lysozyme activity, and lower concentrations of lactoferrin and thiocyanate.

Considering the combined input of all variables of the unstimulated and stimulated saliva, all three volunteers with very low caries increment again were grouped into one cluster, again together with one volunteer with a low caries increment and fourteen volunteers with a caries increment higher than four, as may be seen in Table 2. The volunteers with very low caries increment were the same as in the first cluster analysis using solely the variables of unstimulated saliva. The significant results of salivary analysis for these two clusters are given in Table 4.

Table 4 Salivary variables with significant group differences for cluster-modeling based on the variables of unstimulated saliva or of unstimulated and stimulated saliva

Variable	Cluster 1	Cluster 2	t-test
	Mean, SD	Mean, SD	P
Unstimulated saliva			
Flow rate (ml/min)	0.33, 0.19	0.19, 0.07	0.008
Lysozyme (mg/l)	6.84, 4.92	67.60, 36.35	<0.001
Lactoferrin (mg/l)	4.18, 2.10	9.33, 6.13	0.028
Thiocyanate (mmol/l)	1.94, 0.91	3.15, 0.91	0.003
Stimulated saliva			
p CO_2 (kPa)	4.03, 1.12	5.33, 1.77	0.027
Lactoferrin (mg/l)	2.981.24	6.42, 4.03	0.003

In addition to the significant differences for flow rate, lysozyme activity, and concentration of lactoferrin and thiocyanate described for the cluster analysis based on unstimulated saliva, here the cluster including the volunteers with very low caries increment additionally had significantly lower results for partial pressure of CO_2 and for lactoferrin in the stimulated saliva**.** No significant difference could be shown for the variables flow rate, pH, final pH with 0.003 M or 0.005 M HCl, buffer bases, bicarbonate, lysozyme, sIgA, peroxidase, thiocyanate, and total protein of the stimulated saliva. The mean values of these variables also are given in Table 3. Further cluster processing with input of variables of stimulated saliva or with secretion rates of these salivary variables did not reveal a separate cluster for volunteers with low caries increment nor another cluster grouping volunteers with very low caries increment.

Discussion

Dental caries is a multifactorial disease. There is no doubt of the bacterial cause of caries lesions, but additional factors or indicators have an influence on acceleration or slowing down of the development of new caries. Caries activity has been analyzed comprehensively in relation to oral hygiene, socioeconomic factors, and microbiological state of the saliva.

Indigenous and acquired immune response is involved in the caries process. Immune factors in the saliva also have an influence on caries initiation and caries activity [29]. It has proven difficult always to demonstrate significant differences of salivary factors between caries-free (or caries-inactive) and caries-susceptible (or caries-active) volunteers in the different studies relating to this topic. Up to now, no single salivary factor could be shown to be strongly associated with caries data [8, 26]. In our study, we tried to contribute to this discussion with the multivariate approach in a cluster analysis starting from salivary variables and then looking at the caries data of various cluster members.

Salivary variables show large variation. Now, no widely accepted normal ranges have been established. It may be stated that our results are within the published ranges

[7, 8, 12, 26, 27, 28]. The influence of circadian variation was reduced by sampling at the same time of the day. All salivary specimens were collected within 1 month. Thus, a possible circannual variation of salivary data should be only of minor importance. All participants belonged to the same age group, reducing the influence of age on salivary results. In all, the data should be sufficient for analysis of the interrelationship of saliva with caries data.

The study was done to get information about a relationship between caries increment in young adults over a longer period of 4 years and salivary composition at baseline. Our study group was fairly heterogeneous, with high variations in the clinical data (Table 2). Only eight volunteers had a low caries increment over 4 years. The cluster analysis failed to group them into one separate cluster. On the other hand, the same three volunteers with very low caries increment were always grouped into one cluster in both analyses but together with volunteers with a higher caries increment within 4 years. Of the analyzed salivary variables, higher flow rate of unstimulated saliva combined with lower activities or concentrations, respectively, of lysozyme, lactoferrin, and thiocyanate were typical for the cluster group including those three volunteers with very low caries increment. These findings are plausible and in concurrence with those of other authors [25].

The coexistence of volunteers with low and high caries increment in one and the same cluster can partly be explained by the statistical method of cluster analysis. This method has to combine and weigh several criteria. Ranges are implicated in this procedure. Furthermore, it must be taken into account that other factors not analyzed in this study might vary more between individuals with high or low caries increment.

It is becoming clear now that saliva has to be considered in its entirety to account fully for its overall effect on teeth [15]. Thus, multifactorial analyses of the interrelationship between salivary composition and caries decay rather than the use of single parameters could improve the foundations for caries risk prediction on the basis of salivary data for individuals or groups [31].

At first sight, it seems contradictory that individuals with the lowest caries increment were included in the cluster with lower baseline values for the antimicrobial salivary components lysozyme, lactoferrin, and thiocyanate. Their antimicrobial potential is well known and has been proven also against the cariogenic micro-organism *Streptococcus mutans* [26, 33]; but most salivary molecules are multifunctional, which results in built-in redundancies with regard to their protective functions [16]. Thus, lower levels of some antimicrobial variables in the saliva can be compensated by changing levels of other molecules, for instance components such as amylase or histatins [1], which were not included in this study.

Another aspect arises from the concept of amphifunctionality, which implies that salivary molecules may have both protective and detrimental properties depending on location and site of action [16]. Furthermore, it must be taken into account that lysozyme and lactoferrin in saliva are produced not only in the salivary glands but also may stem from polymorphonuclear leukocytes via the crevicular fluid [26] and are synthesized by secretory epithelial cells at mucosal sites adjacent to lymphocyte regions [10].

Cluster analysis has seldom been applied to salivary data. With data for resting whole saliva, Jalil et al. [12, 13] identified three groups of subjects with different salivary profiles which they related to the amounts of plaque and the occurrence of gingivitis. The main difference was in the level of sIgA. In a large sample of female and male students aged 17–24, Rudney [22] identified seven major groupings by cluster analysis with the salivary antimicrobial proteins peroxidase, lysozyme, lactoferrin, and sIgA in stimulated parotid saliva, whereas Rudney and Smith [21] found six major clusters on the basis of data for the same salivary components. Both papers did not combine these results with any data on oral health.

Cluster analysis for the relationship between caries and saliva was used in a 2-year study by Kirstilä et al. [15]. Two different salivary profiles in children were found by these authors. All salivary variables tested were significantly different between both clusters, regardless of the caries increment. Our study, one of the longest longitudinal studies on the relationship between caries increment and salivary defense factors, is in agreement with the suggestion that other factors such as lysozyme and lactoferrin in addition to the regulatory role of secretory IgA antibodies, hypothiocyanite, and agglutinins [28] are of importance where synergistic or additive effects can occur [29].

Influences of individuals' general health and defense state [11, 14, 22, 23] can overshadow evidence of the relationship between caries and saliva. More detailed knowledge on the interplay of the defense processes in saliva still is necessary but cannot replace the importance of combining clinical and paraclinical studies in which the overall effect of saliva is taken into account by a multivariate approach.

Conclusions

A multivariate approach by cluster analysis based on salivary data may be useful for unraveling those components from an array of salivary data which are important for a low caries increment. Within the limits of the present study, it may be concluded that individuals with low caries increment over 4 years do not form a separate cluster based on the salivary variables studied but are always grouped together in the same cluster with subjects having a higher caries increment. Otherwise, all subjects with very low caries increment would have been included in this cluster, which is characterized by higher salivary flow rate and lower levels of lysozyme, lactoferrin, and thiocyanate in the unstimulated saliva as well as by lower content of CO_2 and lactoferrin in stimulated saliva.

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